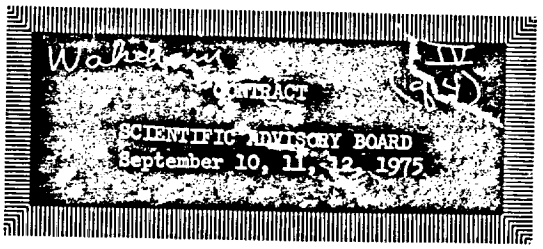


THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

September 2, 1975

To: The Scientific Advisory Board

Subject: Human AHH Studies: A Contract Renewal Proposal From R. E. Kouri, Michrobiological Associates

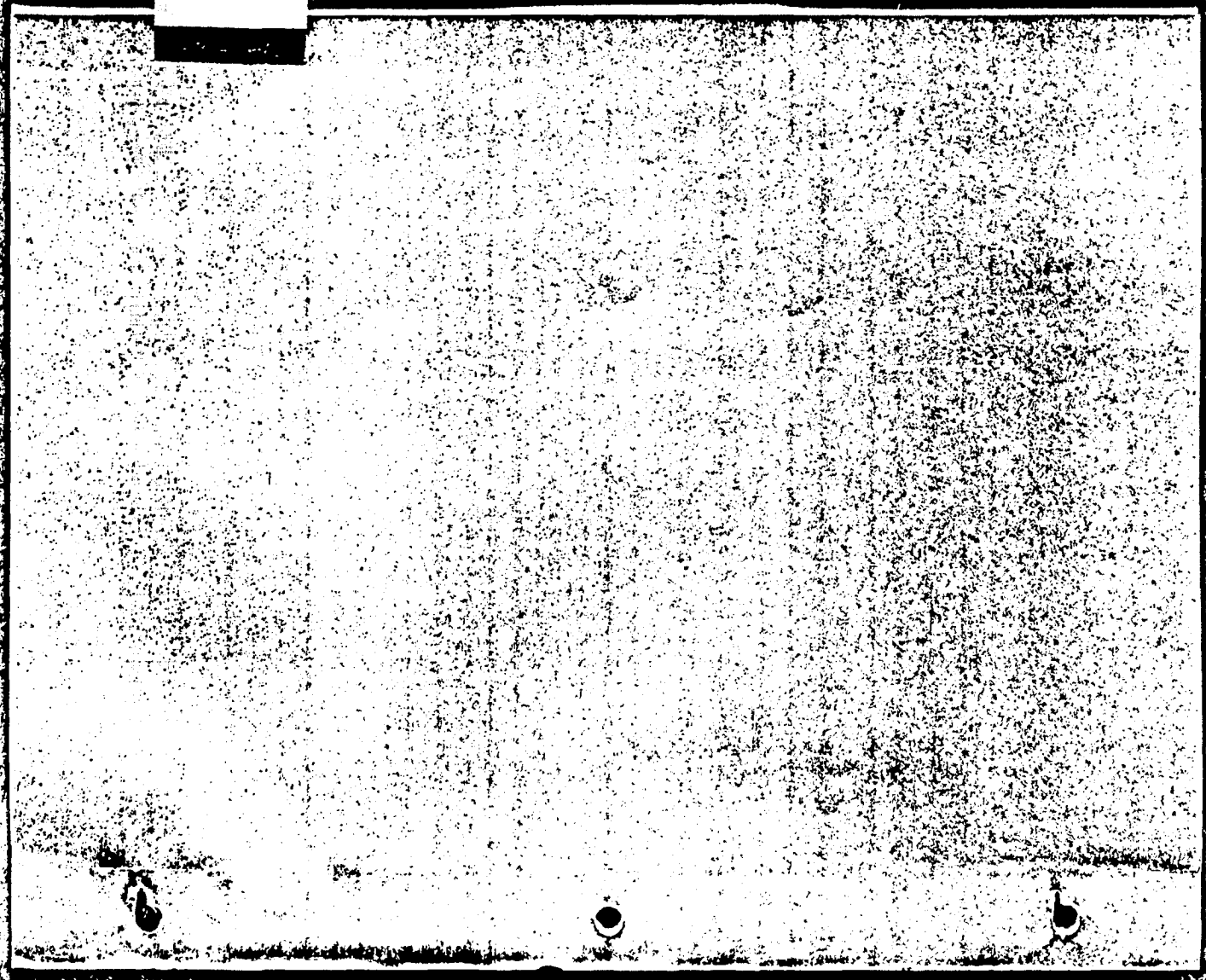
As can be seen in the accompanying progress report a great deal has been accomplished over the past year to standardize the AHH assay to the point that this test could be used to sample lung cancer and control populations. The lymphocyte is one of the few tissues readily available, but the enzyme level is very low unless the cells are in blast state. Because of the difficulties of growing lymphocytes a large day to day variance has been observed. Some progress has been made, and the assay has been improved measurably. However, the reproducibility is still a problem.

Microbiological's Dr.R. Kouri has been a pioneer in AHH genetic studies in animals, and is now a recipient of supplementary contracts from the N.C.I. to study and improve the human AHH assay, along with Dr. T. Yamouchi (also a CTR grantee with Dr. C. Shaw at M. D. Anderson), and Dr. Ken Paigen (Roswell Park). These laboratories are collaborating closely to achieve an adequate testing system. It would seem appropriate to continue this work for another year.

In similar regard it has been suggested that renewal Grant No. 1013R1 to Dr. Malcolm Pike (under Drs. M. Gardner and B. Henderson) of the U. of S. C. be held in abeyance until a successful test system has been developed. A small grant ($10-15,000) to cover U.S.C. staff time in obtaining and shipping lung cancer and control samples for testing reproducibility, field procedures, etc., would be adequate to assure continuity and access. Should the originally proposed human lung cancer - AHH genetic field study become a possibility in the future increased funding could be requested and Scientific Advisory Board approval sought.

JHK:wg

J.H.K.



RESEARCH CONTRACT PROPOSAL
(Renewal of Contract No. 0025)

for the period

January 1, 1976 through December 31, 1976

TITLE: HUMAN AHH STUDIES

TO: Dr. John H. Kreisher
The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division of
Dynasciences Corporation
4733 Bethesda Avenue
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DATE: August 22, 1975

Vincent L. Ruwet
Vincent L. Ruwet
Vice President, Contracts
and Administration

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CONTENTS

I. INTRODUCTION

The determination of the relative risks of particular human populations to cancer in general, and tobacco-associated cancers, in particular, is a major goal in the nationwide program to control cancer in man. During the past year, major effort has been directed toward understanding the role of the enzyme complex, aryl hydrocarbon hydroxylase (AHH), in human cancer - for the level of these enzymes seem to be correlated with susceptibility to cancer in model animal systems, and preliminary evidence suggests the same relationship may hold for the human system as well. The confirmation of this latter relationship may be one of the most important studies presently being undertaken in the field of cancer research.

The confirmation of this relationship has been plagued by problems with the reproducibility of the assay system itself. It is proposed in this study that a reproducible assay can be achieved and that this assay be used to establish the relationship between levels of AHH activity and at least eight different cancers, including cigarette smoke-associated lung carcinomas.

II. BACKGROUND

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component microsomal-bound enzymes (1, 2). The actual steps involved in this oxidative metabolism are unknown; however, the best guess is that the substrate combines with the oxidized form of a carbon monoxide sensitive hemoprotein called cytochrome P-450. The substrate-cytochrome P-450 complex is then reduced by an electron donated by NADPH-cytochrome c reductase to form a reduced substrate-cytochrome P-450 complex. This complex in turn reacts with molecular oxygen to form a reduced substrate-cytochrome P-450-O_2 complex. A second electron is then added to this complex to yield an active oxygen intermediate that decomposes with the formation of the product and the oxidized P-450. The product of this reaction, if polycyclic aromatic hydrocarbons are substrates, are probably epoxides (3, 4). These intermediates then (a) rearrange spontaneously to form phenols, (b) are enzymatically metabolized to the dihydrodiol via the enzyme epoxide hydrase, or (c) are enzymatically conjugated with glutathione using the enzyme glutathione conjugase.

These enzymes have two properties which make them uniquely important to the study of chemical carcinogenesis. First, metabolism of many substrates (especially PAH) does not necessarily result in detoxification, but rather are converted to water-soluble forms via transient chemically-reactive intermediates that are both cytotoxic (5, 7) and carcinogenic (8-10). Second, that this enzyme system is inducible by certain substrates and that this induction results in the enhanced metabolism of many foreign compounds (11). This latter property is important because, if these metabolic pathways for PAH are etiologically important in the initiation of chemical carcinogenesis, any marked changes in rates of formation of the water-soluble metabolites (e.g. epoxides, phenols, dihydrodiols and glutathione conjugates) or covalently bound metabolites should affect the host's susceptibility to cancer.

In the house mouse Mus musculus, not only is AHH induced by treatment with certain substrates, but also this inducibility is host gene regulated. Treatment with phenobarbital (PB) increases the metabolism of most of drug substrates and in every strain of mouse tested (12). Treatment with 3-methylcholanthrene (MCA) increases the metabolism of very few substrates and in only particular strains (13). These differences probably result from the fact that PB causes a rapid non-specific proliferation of constitutive AHH (14), while MCA induces a new spectrally distinct cytochrome called P^1-450 (15) or P-448 (16) which has different substrate specificities. The ability to respond to

MCA (but not PB) segregates as a single autosomal gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (13, 17-19). Thomas et al (13) and Kouri et al (20, 21) propose this locus be designated Ah; the allele carried by the B6 mouse (inducible) is Ah^b, and the allele carried by the D2 mouse (noninducible) is Ah^d. Following treatment with MCA, the difference between the AHH levels in various tissues of Ah^b/Ah^b or Ah^b/Ah^d mice are 2-80 fold greater than that of Ah^d/Ah^d animals (17-21). Thus, one can evaluate tumor susceptibility among litter-mates in which the presence or absence of AHH induction is expressed in their tissues. With the use of such a model, other non-specific strain differences - such as characteristic mouse strain differences involving immunology, latent viral infections, nutrition, hormones, stress, or levels of other enzymes - will be theoretically cancelled.

Using this model system, Kouri et al (20-22) have reported that segregants carrying the Ah^b allele are approximately 12 times more sensitive to MCA induced fibrosarcomas than animals homozygous for the Ah^d allele. Thus, it seems likely that the types of metabolites, or just the quantity of these metabolites determined by this novel "inducible" enzyme play a major role in determining the susceptibility of mice to chemical carcinogenesis.

When studying the mixed-function oxidases in man, two major problems become apparent: the availability of tissue and the availability of sensitive enough assay procedures. Human tissues that have been used include cultured cells in vitro (23, 24), placenta (25-27), foreskin (28), biopsied liver (29-31), skin (32), pulmonary (33, 34), and blood macrophages (35), and peripheral blood lymphocytes (36-38). The available assays include: a) measurement of conversion of BP to 3-OHBP spectro-photofluorometrically (39, 40), b) measurement of conversion of BP to water-soluble forms (24, 41), c) quantitive measurement of conversion of BP to its various metabolites using thin layer chromatography (42-44), and d) cytochemical analysis of individual cells by microfluorometry (45-49), and e) direct quantitation of the P-448 or P-450 cytochromes (30, 50, 51, 52). Only a few investigators have reported data concerning the metabolism of actual chemical carcinogens by human tissue. Kuntzman et al (29) reported that benzo(a)pyrene (BP) was metabolized by autopsied human liver microsomes at a rate similar to that observed in rat liver. Merrill and Campbell (31) reported that aflatoxin was also metabolized by human liver obtained at autopsy. Using cultured human cells, both Huberman and Sachs (23) and Kouri et al (24) have recently shown that these cells can metabolize BP to water-soluble forms, and the rate of metabolizing is influenced by the relative number of epithelial-like cells and perhaps by the age of the cells in culture (number of generations). Recently Bast et al (53) reported that peripheral blood monocytes possessed fairly high AHH activities.

The level of AHH can also be influenced by cigarette smoke. Placental tissue from women who smoke contains much more AHH activity than the tissue from non-smoking women (14, 15).

The system which seems to show the greatest promise in determining the role of AHH in human cancer is the mitogen-activated peripheral lymphocyte. Using this system, Busbee et al (37) and Whitlock et al (36) report that AHH can be detected in both fresh and cultured lymphocytes. Moreover, the spectrum of BP metabolites observed is very similar to that observed from the enzyme in rat liver (52, 54, 55). The extent of inducibility seems variable in the human population (56), and most importantly, these differences may be genetically regulated by an autosomal co-dominant allele (58). The AHH response using other inducers, such as 2, 3, 7, 8-tetrachlorodibenzo-(p)dioxin, follow this same regulation (38). Moreover, the activity in human lymphocytes may, in fact, be correlated with hepato oxidation rates (57).

The recent work by Kellermann et al suggests that this genetically regulated level of AHH may influence susceptibility to cigarette-smoke associated lung cancers, just like genetically-regulated levels of AHH effect susceptibility of MCA-induced tumors in mice (21). Kellermann et al (59) reported that a much higher percentage of medically verified lung cancer patients were either high or intermediate AHH inducers; suggesting that not only are polycyclic aromatic hydrocarbons important causes of cancer in humans, but also, that, as in mice, those individuals with a heightened ability to metabolize these chemicals may be more susceptible to the chemically-induced or "spontaneous" cancers. The confirmation of these studies is now being completed in various laboratories.

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III. EXPERIMENTAL

A. From September 1975 to December 31, 1975

1. Establish standard culture conditions

Studies will continue in determining those conditions yielding the most reproducible mitogen-activation of human lymphocytes. Work will center on evaluating the use of purified mitogens (e.g. leucoagglutinin, Pharmacia Fine Chemicals, New Market, N. J.), non-carcinogenic inducers (e.g. 5, 6-benzoflavone), and particular lots of fetal calf serum as ways to standardize mitogen-activation.

2. Establish methods to correct day-to-day fluctuations in mitogen-activation

Even under the most exact culture conditions, differences in rates of mitogen activation may be observed. This probably results from inherent differences in lymphocyte populations in vivo. Whatever the reason, methods may have to be established to correct these day-to-day variations. It is proposed to continue evaluating rates of DNA, RNA and protein synthesis as a method to correct variations in mitogen activation.

3. Studies with the "standard" lot of FCS from GIBCO

It is proposed to determine the reason why cells cultured in the same FCS, but in different laboratories, give different levels of AHH. In collaboration with Dr. T. Yamauchi (M.D. Anderson Hospital and Tumor Institute, Houston, Texas), blood samples from individuals within our laboratories will be exchanged, activated in both laboratories, and cells will be assayed in both laboratories. Frozen mitogen-activated, induced and non-induced samples will be assayed. Determinations of the factor(s) that cause these disparate levels of AHH activity should go a long way in achieving a more standardized assay system.

4. Determination of standard parameters on which to base AHH activity

Six males (from within our own laboratories) will be assayed once a week for three consecutive weeks. The rates of AHH per ug DNA, per rate of DNA synthesis, and per flask will be evaluated. Also, the total amount of AHH produced in response to co-treatment with mitogens and inducers will be determine. Total

activity will be measured at 48, 72, 96 and 120 hours of mitogen and inducer treatment. The use of rates of incorporation of H^3-thymidine in acid precipitable material as a potential correction factor for variation in day-to-day mitogen activation will also be determined at this time.

A second set of six males will be assayed once a week for three consecutive weeks for observations on the use of levels of NADH-dependent cytochome c reductase as standard parameter on which to base AHH activity. As shown in the progress report, the level of this enzyme seems dependent on the amount of mitogen activation (or amount of microsomes) and independent of treatment with AHH inducers. Thus, the level of this enzyme could be a way to obviate the need for counting cells, quantitating DNA, or determining correction factors for mitogen activation. Preliminary work designed to establish the optimal conditions for measuring this enzyme activity will be done in collaboration with Dr. R. Prough (Southwest Medical School, Dallas, Texas).

5. Determination of relationship between fluorometric and radiometric assay for AHH activity

In collaboration with Dr. H. Guirgis (Creighton University, Omaha, Nebraska), the level of AHH in ten standard individuals in our laboratories will be simultaneously studied by both the radiometric assay and fluorometric assay for AHH activity. The radiometric assay to be used by Creighton University involves the quantitation of the rates of metabolism of H^3-benzo(a)pyrene from an organic-soluble to water-soluble forms by mitogen-activated human lymphocytes. The fluorometric assay will be based on the results of the experiments outlined in the previous sections. The exact same culture conditions, including medium, FCS, mitogens, and inducers, will be used in both laboratories.

B. From January 1, 1976 to December 31, 1976

1. Updating assay procedures

Work will continue on evaluating alterations or additions to any facet of the assay procedure which may allow for a more reproducible, more rapid, or more efficient assay of AHH activity. This will include evaluating a) use of ethoxyresorufin as an alternate substrate for AHH activity (see progress report); b) use of alternate assay procedures, such as quantitation of levels of cytochromes in human lymphocytes; c) use of combination of inducers, such as those that specifically alter constitutive, or P-450 mediated, enzyme activity (e.g. tryptamine, B-estradiol, phenobarbital, aminophylline or theophylline), and those that

specifically alter "induced," or P-448 mediated, enzymes (e.g. MCA, 5, 6-benzoflavone, diphenyloxazole, 5-bromobenzanthracene, etc.), and d) new culture conditions, such as novel purified mitogens, chemically-defined medium, and conditions allowing for preferential activations of specific subpopulations of human lymphocytes.

2. Participation in blind protocol

In collaboration with another laboratory (e.g., Creighton University of USC Medical Center, Los Angeles, Calif.), samples of diluted whole blood (or isolated lymphocytes) will be shipped under code to our laboratory and assayed for AHH activity. Samples will be sent every week for a total of four weeks. These samples will include not only multiple samples from the same individual on the same day, but also samples from the same individual on different days. The code will then be broken and results assessed. At the discretion of the Project Officer, the blind assay will either be repeated or studies into the determination of enzyme levels in cancer patients initiated.

3. Determination of enzyme activity in cancer patients

a. Studies on mitogen-activation of lymphocytes from cancer patients

Preliminary studies will center on the problem of mitogen-activating lymphocytes from cancer patients. It is a well-known fact that lymphocytes from cancer patients respond very poorly to mitogens. It is felt that with just minor modifications of the culture conditions that resulted in optimal growth of lymphocytes from non-cancer patients, that some activation of lymphocytes from cancer patients will be observed. A blind assay, utilizing only cancer patients, may be required. The ability of such a blind protocol will be evaluated at that time.

b. Relationship of levels of AHH activity to cancer susceptibility

These proposed procedures are very similar to those originally proposed for this contract, but because of assay difficulties, could not be completed. The population of Menck et al is a good example of the population that will be used because it appears to fulfill the criteria of accessibility as well as availability of adequate numbers of patients. The intial study will be concentrated on the lung cancer population. Fifty to 100 lung cancer patients, 50 to 100 hospital controls and 50 to 100 non-hospitalized controls will be assayed.

Assays will be done at MA at a rate of 28 to 50 per week. This study will also entail a limited questionnaire involvement: including history of cigarette smoking, drug exposure, occupation, etc. Medical verification of the pathological lesion will also be done.

The suggested approach will concentrate on the importance of cancer association and AHH levels. A matched population would seem to be the best obtainable control at this level. The requirement for detailed questionnaire and multivariant analysis of same must be incorporated to observe correlates and associations. Smoking habits, while normally considered a tertiary study variable, are included in this secondary study, since this variable has particular interest to the granting agency. The procedure for this secondary study will include one hundred patients for each of seven cancer types (total 700) and 700 to 800 controls matched for age, sex, ethnic group and smoking habits. The routine fluorometric assay discussed before will be used. However, concurrent preparations for automated analysis will be included in preparation for the tertiary level studies. The questionnaire for this study will be detailed and organized for computerization. This in-depth level of inquiry would give operational experience and possible leads for specific in-depth tertiary studies. This detailed questionnaire is compatible with the 1,500 patients projected. Areas to be emphasized in this secondary level include: (1) normal medical history; (2) detailed employment history - with master breakdown code of probable PAH, etc. exposure; (3) detailed drug history, weighted for those drugs involved in AHH; (4) detailed smoking history; (5) detailed alcoholic consumption history; (6) other variables to be defined - possible psychological evaluations.

The questionnaire validity will be important. Analysis should be undertaken for possible correlations as the data accumulates. These correlations can be used to predict, direct, and avoid unnecessary duplication in the following studies.

IV. PERSONNEL

A. Dr. R. E. Kouri (Principal Investigator) (10%)
(see original proposal)

B. Dr. Richard L. Imblum (Assistant Project Director)
(40%)

Dr. Imblum received his Ph. D. degree in biochemistry from Purdue University. His doctoral research involved studies on a microsomal enzyme, HMG-CoA reductase, in rat liver and Neurospora crassa. He was a postdoctoral fellow of the American Cancer Society at the University of Virginia where his studies concerned the biochemical processes involved in the replication of vesicular stomatitis virus.

Dr. Imblum has had extensive experience in culturing mammalian cells, general enzymology, and mammalian molecular macromolecules including phenol extraction, column chromatography, rate zonal and equilibrium density, ultracentrifugation, polyacrylamide gel electrophoresis of protein and nucleic acids, thin-layer chromatography, paper electrophoresis, and autoradiography.

A copy of Dr. Imblum's curriculum vitae is attached.

Soc. Sec. No. 521-42-5682

CURRICULUM VITAE - RICHARD L. IMBLUM

BIRTH: REDACTED

EDUCATION:

1972	Ph. D.,	Biochemistry Purdue University Lafayette, Indiana
1966	M. S.,	Biochemistry The George Washington University Washington, D. C.
1962	B. A.,	Chemistry University of Colorado, Boulder

PROFESSIONAL AFFILIATIONS: American Society for Microbiology
American Association for the Advancement of Science

PRESENT POSITION: 1975 - present REDACTED

POSITION DESCRIPTION: Contract with the National Cancer Institute, Standardization of Aryl Hydrocarbon Hydroxylase Assay, as a Screening Method to Determine Smoking Hazards in Man; Contract with The Council for Tobacco Research, The Role of Aryl Hydrocarbon Hydroxylase in Human Cancers.

PRIOR EXPERIENCE: 1972 - 1975 REDACTED

1968 - 1972 REDACTED

1967 - 1968

REDACTED

1962 - 1967

REDACTED

PUBLICATIONS - RICHARD L. IMBLUM

Imblum, R. L., Prosky, L., Libby, D. A., O'Dell, R. G., and Roberts, B., Jr. Effects of 2-Deoxy-D-glucose on Nucleic Acids of Rat Liver. Federation Proc., 26: 798, 1967.

Prosky, L., Roberts, B., Jr., O'Dell, R. G., and Imblum, R. L. Differential Effects of Actinomycin D on Nucleic Acid and Protein Synthesis in Rat Liver. Arch. Biochem. Biophys., 126: 393-398, 1968.

Shapiro, D. J., Imblum, R. L., and Rodwell, V. W. Thin-layer Chromatographic Assay for HMG-CoA Reductase and Mevalonic Acid. Anal. Biochem., 31: 383-390, 1969.

Shapiro, D. J., Imblum, R. L., McNamara, D. J., and Rodwell, V. W. A TLC Assay for HMG-CoA Reductase. Properties of the Rat Liver Microsomal Enzyme. Eastern Analytical Symposium, American Chemical Society, New York, p. 30, 1969. (Abstract)

Imblum, R. L., and Rodwell, V. W. 3-Hydroxy-3-methylglutaryl-CoA Reductase and Mevalonic Kinase of Neurospora crossa. J. Lipid Res., 15: 211-222, 1974.

Imblum, R. L., and Wagner, R. R. Protein Kinase and Phosphoproteins of Vesicular Stomatitis Virus. J. Virol., 13: 113-124, 1974.

Wagner, R. R., Emerson, S. U., Imblum, R. L., and Kelley, J. M. Structure-Function Relationships of the Proteins of Vesicular Stomatitis Virus, in "Negative Strand Viruses." (R. D. Barry and B. W. J. Mahy, Ed.), Academic Press, London, 1974.

Imblum, R. L., and Wagner, R. R. Inhibition of Viral Transcriptase by Immunoglobulin Directed Against the Nucleocapsid NS Protein of Vesicular Stomatitis Virus. J. Virol., 15: 1357-1366, 1975.

V. BUDGET

A.	Total Direct Labor (See Schedule A)	$ 27,138
B.	Overhead (115% of A)	31,209
C.	Other Direct Costs (See Schedule B)	15,700
D.	Travel ($500/professional plus one trip Los Angeles - Washington, D.C.)	1,500
E.	Total (A - D)	75,547
F.	G & A (16% of E)	12,088
G.	Total Costs	87,635
H.	Fixed Fee (10%)	9,737
I.	Total Cost Before Equipment	97,372
J.	Equipment (See Schedule C)	9,700
K.	Total Cost	$107,072

SCHEDULE A - TOTAL DIRECT LABOR

Name	Function	Time on Project	Total Hours*	$ Hour	$
R.E. Kouri, Ph.D.	Project Director	10%	193	REDACTED	
R. Imblum	Assistant Project Director	40%	770	REDACTED	
C. McKinney	Senior Technician	100%	1,926	REDACTED	
R. Sosnowski	Technician	100%	1,926	REDACTED	
	Total Hours		4,815		
	Total Direct Labor				25,602
	+ 6% Raise				1,536
	TOTAL DIRECT LABOR				$27,138

SCHEDULE B - OTHER DIRECT COSTS

Materials:

Media	$3,000	
Blood samples	500	
Chemicals	5,500	$9,000

Expendable Supplies:

Glassware (production and reuseable)	2,700	
Disposable glassware	4,000	6,700

TOTAL OTHER DIRECT COSTS $15,700

SCHEDULE C - EQUIPMENT

Scintillation Counter, LS 100, 3 Channel, 60 Hz.	$9,500
Source: Beckman Instruments, Inc.	
Plus 2% Sales Tax.....	190
TOTAL EQUIPMENT	$9,690
Amount per Budget	$9,700

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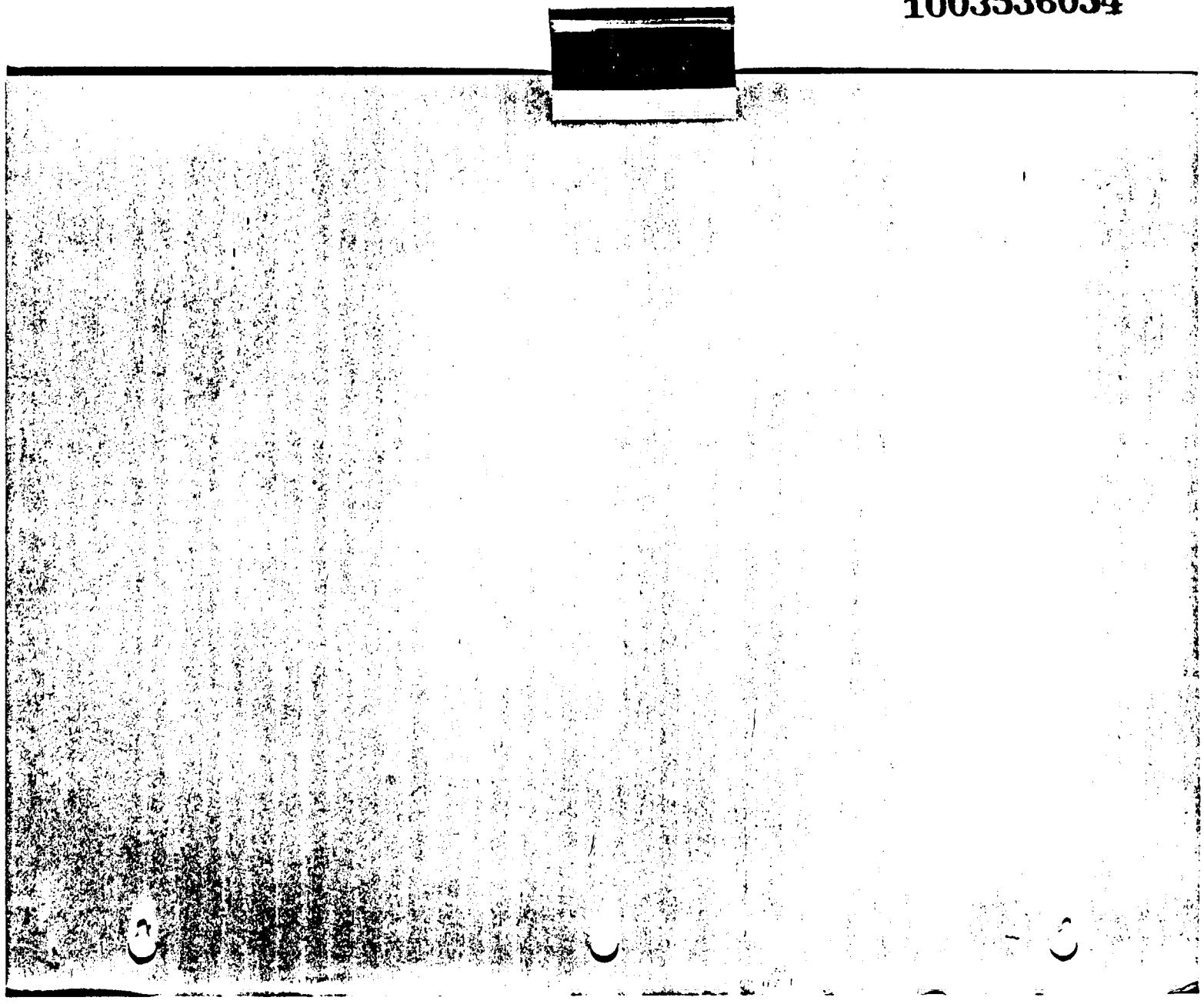

Progress Report

from the period

November 1, 1974 through August 1, 1975

Contract No. 0025

from

The Council for Tobacco Research

"Human Aryl Hydrocarbon Hydroxylase Studies"

Microbiological Associates August 15, 1975
Annual Report
CTR Contract 0025

I. Work accomplished, Milestones

A. Isolation of human lymphocytes.

Table 1 presents our step-by-step procedure for the isolation of human lymphocytes using ficoll-hypaque gradients. The ficoll-hypaque solution is now available from Microbiological Associates as a sterile working solution pre-tested to have a density of 1.08g/ml. Our normal yield of blood lymphocytes using this procedure is about 1.0×10^6 cells/ml whole blood. As measured by the Wrights stain, the isolated white blood cells are at least 90-95% small lymphocytes. Because of their rather uniform size, an automatic cell counter, such as the Fisher Autocytometer II, can be readily used to adjust the cell concentration to the required level.

B. Mitogen activation of human lymphocytes.

The reproducible activation of these isolated lymphocytes is the single biggest problem in the standardization of procedures designed to quantitate aryl hydrocarbon hydroxylase (AHH) activity in these cells. Figure 1 depicts the growth curve of mitogen activation of human lymphocytes when grown in 10 X 75mm test tubes. The optimal conditions are given in table 2. These are our "optimal" conditions; resulting in a 0.5-2 fold increase in cell number during the 96 hour incubation period. Activation is more efficient using 12% autologous serum and the 10 x 75mm test tubes, however, the 10-50% increase in activation observed, is more than offset by the physical impossibility of handling 2 culture tubes for every ml of whole blood. Therefore, the procedure of activation presently being done uses no autologous serum and 25 cm^2 tissue culture flasks.

The effects of various culture conditions on the mitogen activation of human lymphocytes are shown in tables 3-5. Table 3 demonstrates the effect of culture vessel, table 4 shows the effect of various lots of FCS, and table 5 depicts the effects of different sources and types PHA on lymphocyte growth in culture. The growth kinetics of human lymphocytes exposed to different concentrations of PHA-P are shown in figure 2. It is quite ob-

vious that all of these parameters can definitely influence the number of cells responding to mitogen activation.

Another major problem which manifested itself during these studies is the relative difficulty of reproducibly counting activated lymphocytes. Figure 1 shows that the hemocytometer and autocytometer seem to reproducibly count cells for up to 72 hours of activation, but large discrepancies exist for 96 hour cultures. We believe the hemocytometer count to be more accurate because the cell counter cannot discriminate clumps of cells, dead cells, unactivated cells, or small cellular debris from intact lymphoblasts. Thus the hemocytometer is the method of choice for enumerating cultured lymphoblasts, but even this technique has definite shortcomings. The inherent error, even if 100-200 cells per 10 fields are counted, and great care is used, is about 10-15%. Moreover, the presence of clumps of cells, dead cells, and non-activated cells increases the error tremendously. The hemocytometer may be used if only 2-4 assays per week are attempted, but if 8-10 assays per day are required (which will definitely be the case), the hemocytometer is completely unworkable. For example, in order to assay 10 individuals (in triplicate) in one day, 120 separate hemocytometer counts would be required (10 people X 3 samples X 2 parameters (induced and non-induced) X 2 separate counts). To do this number of cell counts plus a careful assay in one day is barely feasible.

A possible alternative to cell counts is total DNA per culture. Figure 3 shows the growth curve and the DNA content of mitogen activated and non-activated human lymphocytes grown in Falcon 3013 flasks. The population of non-activated cells remains constant indicating no cell multiplication when measured either by cell number or total DNA (diphenylamine method). For mitogen-activated cells, DNA content and cell number parallel one another fairly well. Both show an initial drop before cell proliferation. Thus, DNA per culture gives an accurate measurement of the total viable cells per culture but not necessarily a measure of the degree of activation of the lymphocytes.

C. Assay for AHH activity in mitogen-activated human lymphocytes.

With only minor modifications of our existing AHH assay system, we were able to detect and quantitate the enzyme activity associated with cultured human lymphocytes. The modifications were:

1) use of scintillation grade acetone and hexane as solvents,
2) use of deionized water of at least 18 mohm grade, and
3) use of Wheaton disposable 13 X 125 mm glass test tubes as assay vessels.

Under these conditions, the signal to noise ratio (MCA-induced fluorescence values per zero time control fluorescence values) is usually between 40 and 100. Using this modified assay, AHH activity can be detected in cultured human lymphocytes (table 6), the pH optimal for this activity is about 8.5 (table 7), activity is stable to freezing at -70° for at least 3 weeks (table 8), and enzyme activity in vitro is linear for at least 1.0 hour (figure 4). Table 9 shows AHH activity in lymphocytes from two individuals grown in the presence of PHA and PWM either alone or in combination. It is apparent that the two mitogens do not act synergistically nor are their effects additive. In figure 5, the effect of time of MCA induction on AHH activity is shown. Data suggest that the standard 24 hour induction period is less efficient in inducing AHH activity than treatment for 48, 72, or 96 hours. Highest activity is observed if MCA is added simultaneously with mitogens.

Of major importance is the fact that the inducibility ratios observed are not like those reported by Kellermann et al, nor those originally observed in our laboratory (that is, inducibilities of 2, 3 or 4 fold), but rather are in the order of 6 to 15 fold. Our new procedures allowing for optimal growth conditions must be the cause of these differences. The major reasons for these discrepancies are the very low constitutive (or non-induced) AHH activities now associated with these lymphocytes. We suggest that there is really no constitutive AHH activity in migoten-

treated lymphocytes, but rather, the AHH level observed results from the non-specific response of these lymphocytes to contaminants in the culture medium. In this way, the lot-to-lot variations observed for FCS probably result from the presence of various levels of contaminating "inducers" of AHH activity. Data presented by Kellermann et al, and Paigen et al at the recent Orlando, Florida meetings tend to support this hypothesis and Kellermann feels that this "inducer" is time-labile and only relatively young pools of FCS contain this activity. However, we recently obtained the lot number of FCS from Paigen's group which gave inducibilities of 2, 3 and 4 fold in their laboratory. This serum, when tested in our laboratory with our own standard serum as a control, gave the results shown in table 10. The data indicate that with our method of culturing lymphocytes and assaying AHH activity, the two sera give about the same inducibilities, i.e. inducibilities between 6.0 and 9.0. The results, using the two activation procedures (using the 10 X 75mm test tubes which we described in earlier reports or the Falcon #3013 flasks as described by Gurtoo et al (Cancer Res. 35: 1235, 1975)) and a blind protocol are given in tables 11 and 12 respectively. Generally the data indicate that the assay yields reasonable duplicates for a blood sample from one individual if samples were taken and assayed on separate days. Therefore, our culture conditions which seem to result in reliable and efficient cell activation have resulted in rather poor constitutive AHH activity, and it necessarily follows, a rather irreproducible inducibility ratio.

We recently concluded a study of 9 standard males in our laboratory in which blood samples from each test individual were drawn, cultured, and assayed weekly for four consecutive weeks using the RPMI method. Table 13 gives the results, expressed as AHH units per flask, per mg DNA, and per 10^6 viable lymphocytes, for a representative individual. Variations exist no matter which parameter is used to express non-induced and induced activities.

D. Use of frozen lymphocytes.

Table 14 demonstrates that human lymphocytes can be slow-frozen (using a step freezer) and stored at -70° for up to 2 weeks and subsequently assayed for AHH activity. The frozen cells can be thawed (with about a 50% drop in viability), mitogen-activated for 72 hours, induced with MCA for 24 hours, and then assayed for AHH activity. Results indicate that AHH activity can be detected in these frozen-mitogen-activated cells, but the level is different from freshly cultured blood. This difference may not mean anything because we observe these same differences between two freshly cultured samples. Of importance is the fact that lymphocytes from populations that may be unavailable at a later date can perhaps be taken and frozen now, and subsequently assayed at a later time.

E. Use of chemically-defined medium.

The problems with lot-to-lot variation with FCS could perhaps be circumvented by the use of serum-free medium. This approach has worked well with tissue culture cells (Higuchi and Robinson, In Vitro 9: 114, 1973). The AHH activity of human lymphocytes cultured in a chemically-defined medium is shown in table 15. Poor induced and non-induced activity is observed in these cultured cells even if the medium is supplemented with up to 5% FCS. Some activity, however, is observed, and culture conditions will probably require modification in order to effect good mitogen-activation.

F. Use of other reference standards for determination of AHH activity.

The utility of presenting the enzyme activity in terms of AHH units per 10^6 cells has its obvious problems. To devise an alternate reference standard, two requirements seem necessary: (a) that total cellular content be easily and reproducibly quantitated, and (b) that some method for the deduction of the degree of mitogen-activation be used. We are presently evaluating methods to fulfill these requirements. Total cellular con-

tent is being measured by quantitation of total DNA, using the diphenylamine method. Degree of activation is measured by observing the total DNA synthesis (^{3}H-thymidine uptake) occurring during the 96 hour culture period or during the final 24 hours of the culture period. Both total DNA and total DNA synthesis can conveniently be measured on the same sample (table 16). Figure 6 gives data from a recent experiment and shows the relationship between AHH activity, total cell DNA, and ^{3}H-thymidine incorporation into DNA as a function culture time. It looks as if the level of MCA-induced AHH parallels the rate of DNA synthesis.

An alternate approach to expressing AHH activity may be the use of the rate of induction of enzyme activity. Figure 7 shows the rate of induction of AHH from two individuals. If this is reproducible, with each person possessing a characteristic induction curve, then the slope of such a curve, or the total area under the curve, may be valid methods of categorizing individuals.

From the study with 9 standard males (see section C and table 13) we reached the tentative conclusion that data might better be expressed in terms of the induced specific activity, rather than inducibility. When the inducibilities (average values of twelve determinations) are plotted as a distribution, one obtains the pattern shown in figure 8, and when induced specific activities are plotted, one obtains figure 9. There seems to be no difference between individuals if AHH activity is expressed as inducibilities. However, if expressed as just units of AHH activity per mg DNA, three groups are observed. The ranges of these specific activities would certainly overlap, nonetheless, it is interesting that some differences were observed. Moreover, individuals such as GG, TR, and HR, have historically been higher inducers.

G. Use of another assay system to measure mixed function oxidase activity in human lymphocytes.

Measurement of another P-450-dependent enzyme activity in cultured human lymphocytes, O-deethylation of ethoxy-

resorufin is summarized in table 17. This assay could be a very feasible way to measure the level of MCA-induced microsomal enzyme activities because: (a) there seems to be only one end product of enzyme activity, resorufin; (b) the activity can be measured directly with no extraction steps, and (c) the assay would be amenable to automation. This work is being done in collaboration with Dr. M. C. Burke (Karolinska Institute, Stockholm, Sweden).

H. Use of levels of NADH-cytochrome C reductase as a parameter on which to base AHH activity.

In collaboration with Dr. R. Prough (Southwest Medical School, Dallas, Texas), another microsomal enzyme has been studied that could possibly be used as a parameter on which to base AHH activity. A summary of the properties of this enzyme is given in table 18. Of importance is the fact that the level of this enzyme seems dependent on the degree of mitogen activation (figure10), and not on MCA treatment. Thus, the level of NADH-cytochrome reductase may reflect the absolute amount of microsomes in mitogen-activated cells and could serve as a basis on which to compare AHH activities from separate individuals. This procedure would obviate the need for either counting viable cells or determining total DNA in cultured human lymphocytes.

II. Work to be accomplished

A. Evaluation of culture conditions to give reproducible cell growth, including use of different lots of FCS, use of chemically defined medium, use of purified mitogens with reproducible activating properties, use of other inducers of constitutive AHH (e.g. estradiol or tryptamine), and use of other inducers of "induced" AHH (e.g. benz(a)anthracene, 5,6-benzoflavone, or diphenyloxazole).

B. Evaluation of methods to correct for day-to-day variations in lymphocyte activation by the use of isotope incorporation into cell macro-molecules.

C. Repeat assays of cells cultured in GIBCO FCS and attempt to determine why inducibilities we obtained differ from those found in Kellermann's and Paigen's laboratories.

D. Evaluation of O-deethylation assay as a method for determining MCA-induced mixed-function oxidase activity in human lymphocytes.

E. Evaluation of rates of NADH-cytochrome C reductase as a parameter on which to base AHH activity.

Table 1

Procedures for the Isolation of Lymphocytes from Whole Human Blood

1. The area where blood is to be taken is washed with a sterile prepodyne swab (Clinipad Corporation, Stamford, Connecticut).

2. Venous blood is collected in sterile 150ml evacuated containers (McGaw Laboratories, Milledgeville, Georgia) in which 1,000 units of sodium heparin have been previously added.

3. The blood is diluted 20% with sterile HBSS supplemented with penicillin and streptomycin.

4. In 16 x 100 mm plastic tubes, 6 ml of a solution of ficoll-hypaque (s.g. 1.080) is added.

5. 9 ml aliquots of the diluted blood are added to the 6 ml gradients.

6. The ficoll-hypaque gradients are spun at 1500 rpm (590 xg) for 40 minutes.

7. The lymphocyte band (at the interface between the ficoll-hypaque and plasma) is collected with sterile Pasteur pipettes and transferred to 50 ml centrifuge tubes.

8. Cells are washed twice with 25 ml aliquots of RPMI 1640 and cells are counted in an Autocytometer II (Fisher Scientific, Pittsburgh, Pennsylvania) and adjusted to a concentration of 0.5×10^6 cells/ml.

9. The complete medium is RPMI 1640, supplemented with 15% fetal calf serum 0.025M HEPES, 50 units penicillin per ml, 50 μg streptomycin per ml, 1% phytohemagglutinin-M (Difco Laboratories, Detroit, Michigan), and 1% pokeweed mitogen (Grand Island Biological Company, Grand Island, New York).

10. One ml aliquots of cells are added to 10 x 75 mm plastic culture tubes, or 8 ml aliquots are added to Falcon #3013 flasks and in either case, incubation is carried out at 37°, 5% CO_2 - 95% air.

Table 2

Optimal Conditions for Mitogen-Activating Human Lymphocytes.

1. Medium

   a. RPMI - 1640
   b. 15% FCS (Lot # 86629 or 86929) (MA)
   c. 12% autologous serum
   d. 0.025M HEPES
   e. 50 units penicillin/ml, 50µg streptomycin/ml
   f. 1% - PHA-M (Lot # K9268) Burroughs-Wellcome, Inc.
   g. 1% - PWM (Lot # A 045502) GIBCO

2. Vessels

   a. 10 x 75 mm plastic tubes (Falcon)
   or b. $25cm^2$ tissue culture flasks (Falcon)

3. Cell Concentration

   a. 0.5×10^6 cells/ml in a total of 1.0ml/tube
   b. 0.5×10^6 cells/ml in a total of 8.0ml/flask

Table 4

Effect of various lots of FCS on mitogen activation of human lymphocytes.[a,b]

	Lot #	# cells/ml at 96 hours (X 10^6)	Relative Effects
1.	84929	1.40	.93
2.	84970	1.36	.97
3.	82757	1.30	.93
4.	86539	.88	.63
5.	86629	.80	.57

[a]Cells were seeded at 0.5 X 10^6 cells/ml in 12 X 75mm tubes.

[b]Medium was RPMI supplemented with the various FCS at a concentration of 20% (v/v).

[c]Source of lymphocytes was one individual and all assays were done on the same day.

Table 5

Effects of various sources and types of PHA on mitogen-activation.[a]

Mitogen		# cells at 96 hours (X 10^6)	Relative Effect
1. Burroughs-Wellcome	PHA-M	.80	1.00
2. Difco	PHA-M	.65	.81
3. Burroughs-Wellcome	PHA-P[b]		
	1.25	.93	1.16
	2.50	1.04	1.30
	5.00	.16	0.20

[a]Cells seeded at 0.5 X 10^6 cells/ml in 12 X 75mm tubes. Cells were from one individual and all assays were done on the same day.

[b]µg PHA-P/ml culture medium.

Table 6

AHH inducibilities from human lymphocytes grown under our "best conditions".

Subject	N[a]	I[b]	Inducibility
EP	.02[c]	.11	5.5
GH	.02	.29	14.5
TR	.04	.55	14.0
RK	.02	.12	6.0
RS	.02	.12	6.0
RM	.02	.13	6.5
CM	.02	.38	19.0
HR	.03	.24	8.0

[a]Noninduced activity.

[b]MCA-treated cells.

[c]Values given in terms of pMoles 3-OHBP per 10^6 cells per min at pH 8.5.

Table 7.

Effect of pH of Incubation Buffer[a,b]

Source	7.8	8.1	8.3	8.5	8.7	9.0
DA (ind.)	0.046 (3.3)	0.069 (3.0)	0.096 (3.8)	0.100 (3.6)	0.108 (219)	0.092 (2.7)
(n.i.)	0.014	0.021	0.025	0.032	0.037	0.034
GG (ind.)	-	-	0.113 (3.7)	0.143 (3.9)	0.166 (3.6)	0.166 (4.0)
(n.i.)	-	-	0.030	0.037	0.046	0.410

[a]Values given in terms of pMoles 3-OH BP per 10^6 cells per min. The assay was run for 45 minutes at 37°C and each tube contained 4 X 10^6 lymphocytes.

[b]ind. = MCA treated cultures; n.i. = non-induced controls.

Table 8

Effect of storage at -70°C of mitog en-activated, MCA induced cells on AHH activity.[a]

Source	Fresh		Frozen[b]		Frozen	
	N	I	N	I	N	I
SG (10.9)[c]	0.011	0.120	0.012	0.110	0.012	0.120
DA (5.6)	0.045	0.253	0.040	0.240	0.032	0.230
RK (8.7)	0.021	0.182	0.022	0.190	0.028	0.210
IS (11.5	0.060	0.691	0.056	0.676	—	—

[a]Data given in terms of pMoles 3-OHBP per 10^6 cells per min. at pH 8.5.

[b]Cells were stored at -70°C as a pellet for at least 48 hours before assay.

[c]Relative inducibility is given parenthetically.

Table 9

Effect of PHA and PWM on AHH activity in lymphocytes

	Non-induced		Induced	
	1[a]	2[a]	1	2
PHA[b]	0.73[d]	0.75	24.0	11.0
PWM[c]	0.70	0.60	2.7	2.2
PHA + PWM	0.44	0.98	14.2	6.9

[a]Two individuals were assayed.

[b]PHA concentration was 1%

[c]PWM concentration was 1%

[d]Data given in terms of fluorescent units per flask; approximately 4 X 10^6 cells/flask.

Table 10

AHH activity in lymphocytes grown in medium containing FCS from either Microbiological Associates or Grand Island Biologicals

	FCS Source					
	Micro			GIBCO		
Subject	N	I	Ind	N	I	Ind
MM	0.75[a]	4.6	6.1	1.0	5.2	5.2
SG	0.91	7.9	8.7	0.84	5.1	6.1

[a]Data given in terms of fluorescent units per flask. Approximately 4×10^6 cells assayed per flask.

Table 11

Assay of AHH activity using blind protocol.

Subject	Date	AHH #	N[a]	I[b]	Inducibility
JA	10/9	103	.030[c]	.17	5.6
	10/9	107	.033	.14	4.3
	10/9	109	.025	.15	6.0
	10/9	111	.047	.21	4.5
EA	10/10	116	.051	.12	2.4
	10/10	117	.053	.16	3.0
	10/10	119	.027	.11	4.1
	10/10	121	.023	.09	3.9
	10/24	153	.053	.74	13.9
	10/24	154	.101	.91	9.0
	10/24	156	.071	.51	7.3
	10/24	158	.044	.53	12.0
MH	10/3	94	no growth	—	—
	10/3	96	no growth	—	—
	10/3	97	no growth	—	—
	10/3	98	no growth	—	—
	10/24	152	.05	.25	5.0
	10/24	155	.06	.33	5.5
	10/24	157	.07	.27	3.9
	10/24	159	.04	.26	6.5
	11/7	190	no growth	—	—
	11/7	191	no growth	—	—
	11/7	194	.05	.30	6.0
	11/7	195	.06	.28	4.7
MC	10/30	162	.19	.44	2.3
	10/30	163	.21	.47	2.3
	10/30	165	.14	.31	2.2
	10/30	167	.16	.39	2.4
RT	10/30	164	.09	.24	2.7
	10/30	166	.12	.36	3.0
	10/30	168	.13	.41	3.1
	10/30	169	.10	.27	2.7
MP	10/2	81	.16	.53	3.3
	10/2	82	.28	.57	2.0
	10/2	84	.15	.42	2.8
	10/2	86	.24	.48	2.0

[a]Noninduced activity.

[b]MCA-treated cells.

[c]Values given in terms of pMoles 3-OHBP per 10^6 cells per min at pH 8.5.

Table 12

Assay of AHH activity using blind protocol and Roswell Park Procedure

Subject	Date	AHH#	N[a]	I[b]	Inducibility
RP	3/3	339	.65	4.4	7.54
	3/3	341	.75	6.1	8.15
	3/3	343	.73	4.2	5.75
	3/3	345	.60	4.9	8.17
RM	3/3	340	.26	1.52	5.87
	3/3	342	.35	1.55	4.43
	3/3	344	.36	1.25	3.48
	3/3	346	.35	1.25	3.58
LM	3/11	355	1.07	11.3	10.6
	3/11	356	1.33	10.0	7.5
	3/11	359	1.09	10.1	9.25
	3/11	360	1.22	11.8	9.65
	2/11	298	0.59	1.95	3.30
	2/11	299	0.33	0.98	2.96
	2/11	301	0.45	0.85	1.88
	2/11	302	0.51	0.81	1.58
MA	3/11	357	1.13	7.3	6.45
	3/11	358	1.02	9.75	9.55
	3/11	361	.766	6.5	8.5
	3/11	362	1.43	4.75	3.33
	2/11	300	0.40	0.46	1.15
	2/11	303	0.41	0.59	1.44
	2/11	304	0.30	0.37	1.23
SM	2/25	331	1.49	12.1	8.12
	2/25	332	1.56	11.15	7.18
	2/25	335	1.18	12.5	10.6
	2/25	336	1.19	9.25	7.8
MH	2/25	333	.79	1.75	2.3
	2/25	334	.60	.82	1.37
	2/25	337	.49	.785	1.6
	2/25	338	.62	2.64	4.25

[a] Noninduced activity.
[b] MCA-treated cells.
[c] In terms of fluorescent units

Table 13

Inducibility of TR based on different parameters.[a]

Assay	# flasks[b]	units/flask			units/mg DNA			units/10^6 cell		
		N	I	I/N	N	I	I/N	N	I	I/N
191	1	.075	.928	12.3	1.20	17.0	14.2	.063	.864	13.7
	2	.187	2.38	12.7	0.94	12.2	13.0			
	3	.216	2.68	12.4	0.98	11.8	12.0			
195	1	.100	.91	9.1	2.0	20.5	10.2	.058	.640	11.0
	2	.160	1.95	12.2	1.30	17.0	13.1			
	3	.280	2.69	9.6	1.80	20.1	11.2			
199	1	.074	1.73	23.5	1.30	32.4	24.9	.030	.448	14.9
	2	.136	3.25	23.9	1.34	27.4	20.4			
	3	.174	4.56	26.1	1.08	29.8	27.6			
203	1	.075	.98	13.1	1.3	16.6	12.7	.238	2.21	9.3
	2	.172	1.82	10.6	1.4	14.4	10.3			
	3	.180	2.40	13.3	1.3	16.0	12.3			

[a] Data given as described in headings of columns.

[b] Number flasks pooled to do assay; and at least duplicate samples assayed.

Table 14

Effects of freezing whole lymphocytes at -70°C prior to assay.[a]

Source	Fresh		Slow Frozen[b]	
	N	I	N	I
SG	0.029	0.081	0.046	0.097
HR	0.046	0.133	0.071	0.200
GG	0.060	0.211	0.064	0.166
RK	0.030	0.060	0.038	0.064
CF	0.060	0.220	0.074	0.130
KT	0.057	0.110	0.057	0.198

[a]Values given in terms of pMoles 3-OH BP per 10^6 cells per min. The assay was run for 45 min at pH8.5 and each tube contained 4×10^6 lymphocytes.

[b]Lymphocytes were isolatated, then slow-frozen; subsequently, the cells were thawed, mitogen-activated, induced and assayed.

Table 15

The Effect of FBS on Induced and Noninduced AHH Activity of Cells Grown in CDM

Sample (MG)	Non-Induced[a]	Induced[a]	Induced/Non-Induced
0% FBS	.14	.39	2.78
1% FBS	.145	.29	2.07
5% FBS	.22	.69	3.13
Control[b]	.91	12.6	13.8

[a]Activity given in terms of fluorescent units per flask (Flask - Falcon Plastics #3013 containing 8mls of media originally seeded with 1.5 X 10^6 lymphocytes per ml.)

[b]Control=0.5 X 10^6 lymphocytes per ml in RPMI 1640 with 20% FBS.

Table 16

DNA Procedure

1. Add 5 - 7 ml cold (4°C) 2% potassium acetate in 95% ethanol to each assay tube containing the assayed cells, pool, and small amount of acetone-hexane.

2. Sonicate with microtip until the precipitate is well dispersed (5 - 30 sec).

3. Centrifuge at 1300 rpm for 20 min at 5° in PR-2.

4. Discard supernatant fraction and drain tubes.

5. Add 1-2 ml of 1 N PCA (depending on DNA concentration) and hydrolyze for 30 min at 85-90°.

6. Centrifuge at 1300 rpm for 30 min at 5° in PR-2 to pellet protein.

7. Remove two 0.10 ml aliquots of supernatant fraction for determination of radioactive DNA. Place each in vial containing 5ml Aquasol and count by liquid scintillation.

8. Take an additional 0.5ml aliquot of the supernatant fraction and add 1 ml of diphenylamine reagent (400 ml glacial acetic acid, 6 ml conc. H_2SO_4 and 6 g diphenylamine) containing 0.1 ml of acetaldehyde (16 mg/ml) per 20 ml of diphenylamine reagent.

9. Make standards with calf thymus DNA at concentrations of 0, 6, 12, 25, 50, and 75 μg/ml.

10. Place in dark for 22 hrs and read absorbance at 600 nM against a reagent blank or a zero time AHH assay. The components of the pool contribute to the absorbance.

Table 17

Summary of data on O-deethylation activity of human lymphocytes in culture

1. Mitogen-activated human lymphocytes will metabolize ethoxyresorufin to resorufin *in vitro*.

2. The assay can be followed directly in a cuvette by quantitating the amount of fluorescent products associated with the presence of resorufin as a function of time in culture at 25°.

3. The activity is pH dependent with an optimum at 8.5.

4. Enzyme activity is substrate dependent with optimal concentration observed to be greater than 0.002mM. A substrate concentration of 0.004mM was routinely used.

5. Activity is dependent on concentration of NADPH. The optimal concentration being 0.2mM.

6. Under these conditions, enzyme activity is linear for up to 30 min.

7. Enzyme activity is dependent upon cell concentration giving a roughly linear response using cell concentrations from 1×10^6 to 8×10^6 cells per assay.

8. Mitogen-activated methylcholanthrene-induced human lymphocyte cultures can be frozen at -90° for up to two weeks and enzyme activity can still be measured.

9. In a survey of nine different individuals, the enzyme activity is preferentially induced by treatment with 3-methylcholanthrene. Enzyme activity in non-induced or control cultures is very low and barely measurable, however, activity from methylcholanthrene-treated cells is easily measured in culture.

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Table 18

Summary of data on NADH dependent cytochrome c reductase activity of human lymphocytes in culture.

1. Cytochrome c reductase has absolute requirement for NADH.

2. Activity is directly proportioned to number of mitogen-activated cells assayed 4-7nMoles cytochrome c reduced/ min/10^6 cells.

3. The pH optimum is 7.7.

4. Mitochondrial inhibitors e.g. KCN or antimycin A have no effect on enzyme activity.

5. Antibody to NADH-cytochrome c b_5 reductase inhibits enzyme.

6. Antibody to NADPH-cytochrome c reductase has no effect.

7. Antibody to cytochrome b_5 inhibits enzyme.

8. Activity is similar for 4 different people and is similar regardless of MCA treatment.

Fig. 1 Time dependent increase of human lymphocytes during mitogen activation; (●——●) cell counts made with hemocytometers; (○——○) cell counts made with Fisher Autocytometer II.

Fig. 2 Effect of various concentrations of PHA-P on lymphocyte activation in culture

●——● = 0μg/ml □——□ = 1.25μg/ml

○——○ = 2.5μg/ml ■——■ = 5.0μg/ml

Fig. 3 DNA content (- - - -) and number of live cells (—————) in mitogen activated (●) and non-activated (○) lymphocyte cultures. Cell counts were made with hemocytometers; total DNA was determined by the diphenylamine method.

Fig. 4 In vitro metabolism of BP as a function of time. Lymphocytes were mitogen-activated for 72 hrs. and subsequently induced with MCA for 24 hrs. The relative inducibilities are given parenthetically.

Fig. 5 Effect of time of MCA induction on AHH activity. Cultures were induced with MCA at 0, 24, 48 and 72 hrs. (indicated by arrows) and assayed at 24 hr intervals as shown.

Fig. 6 MCA-induced AHH activity (○), (^{3}H)thymidine incorporation into DNA (●), and total DNA by the diphenylamine method (△). Cells were induced with MCA and labeled with (^{3}H) thymidine 24 hrs prior to assay. AHH activity, ^{3}H labeled DNA, and total DNA were assayed in the same cells in duplicate.

Fig. 7 Rate of increase of MCA-induced AHH activity for two individuals. All cultures were induced with MCA at 0 hrs. and assayed at designated times.

Fig. 8 Relative inducibilities of 9 individuals assayed for AHH activity at 3 different times. Each plot represents the average of 12 determinations.

Fig. 9 Specific activity of AHH of 9 individuals assayed at 3 different times. Each plot represents the average of 12 determinations.

Fig. 10 NADH-cytochrome c reductase activity and ^{3}H thymidine incorporation into DNA in PHA and pokeweed mitogen-stimulated lymphocytes. Cells were induced (X) or not induced (●) with MCA at 0 time and labeled with ^{3}H thymidine every 24 hrs. At the end of each 24 hrs. period cells were assayed for cytochrome c reductase, DNA, and ^{3}H thymidine incorporation.

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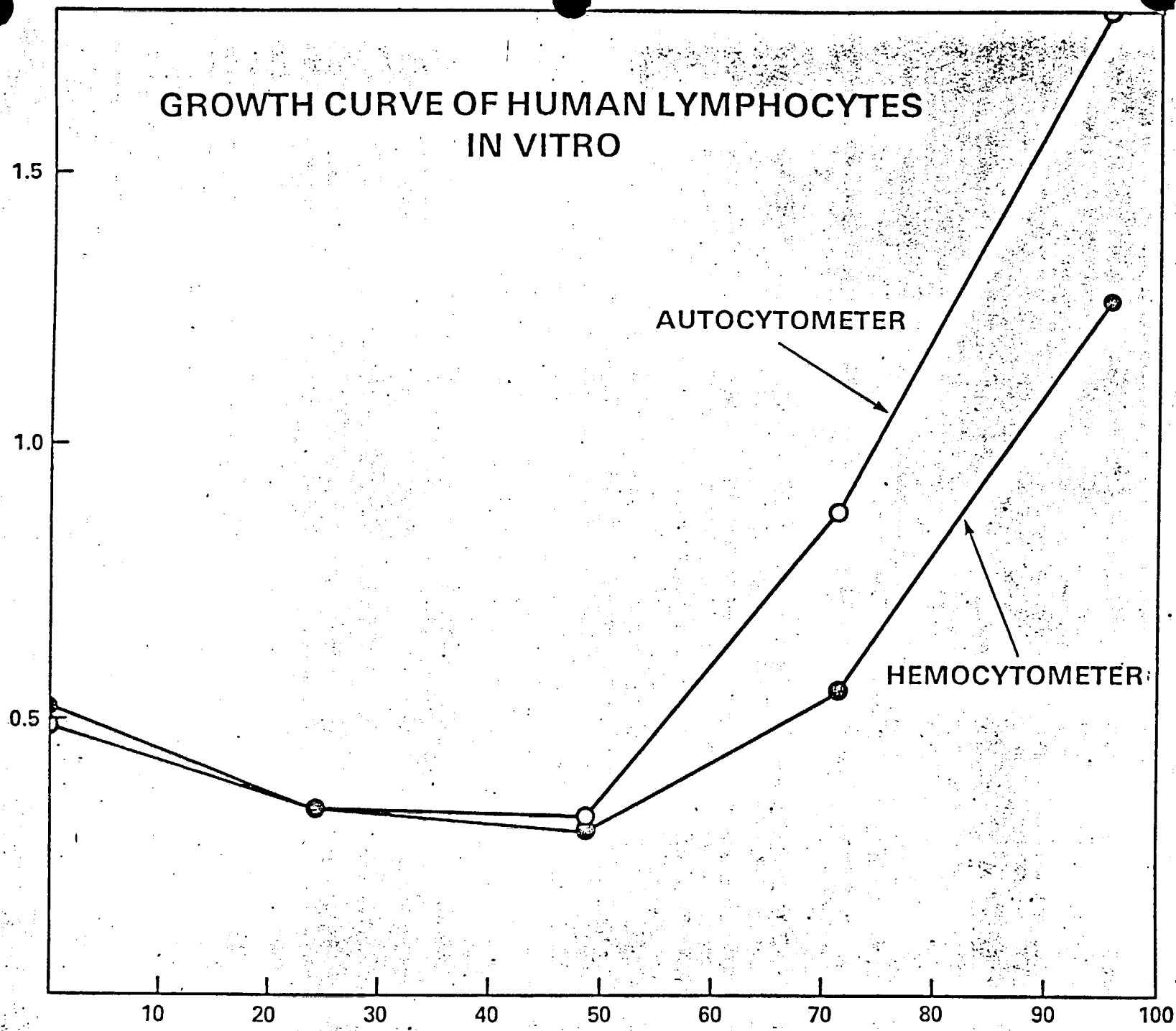

Figure 1

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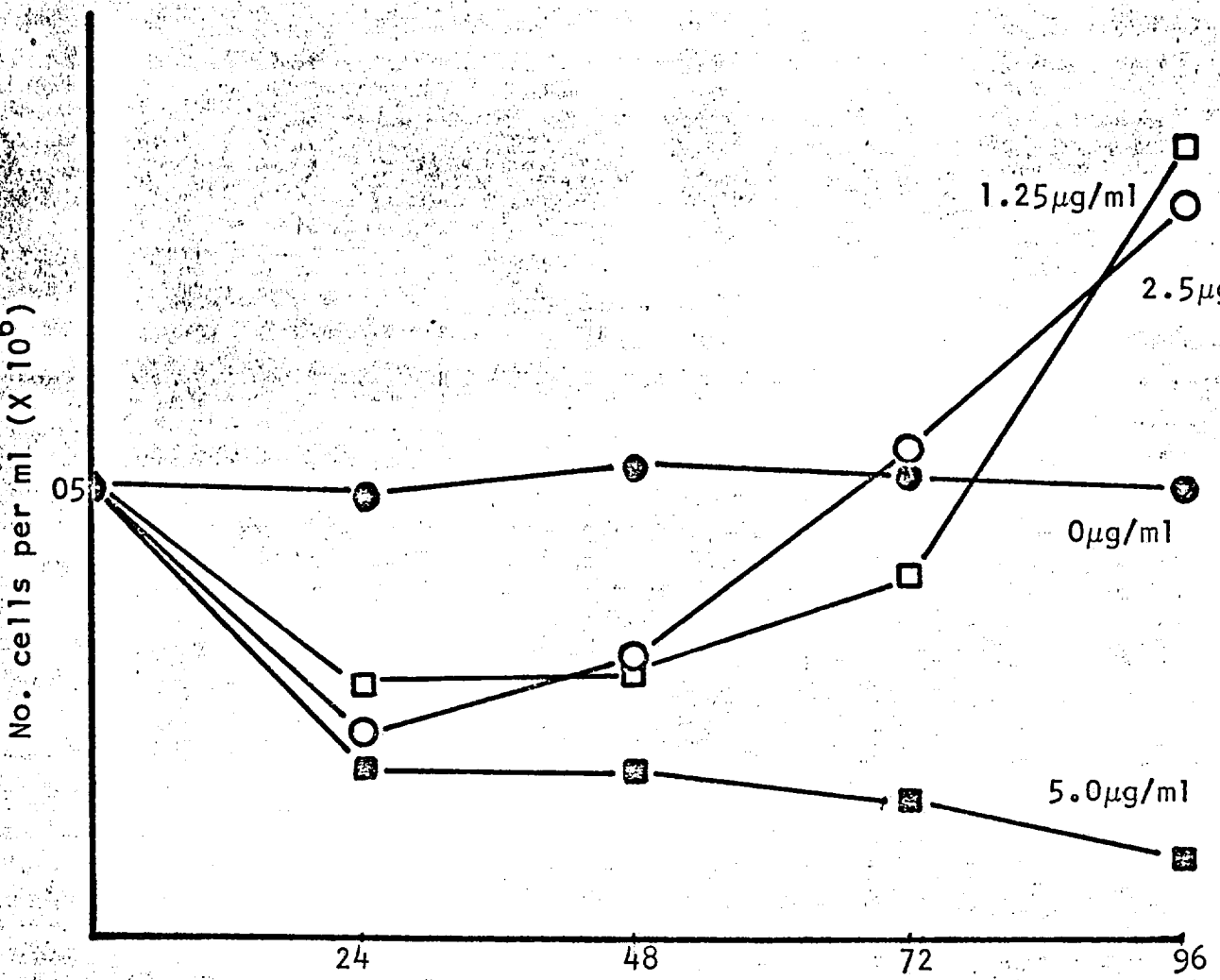
Figure 2



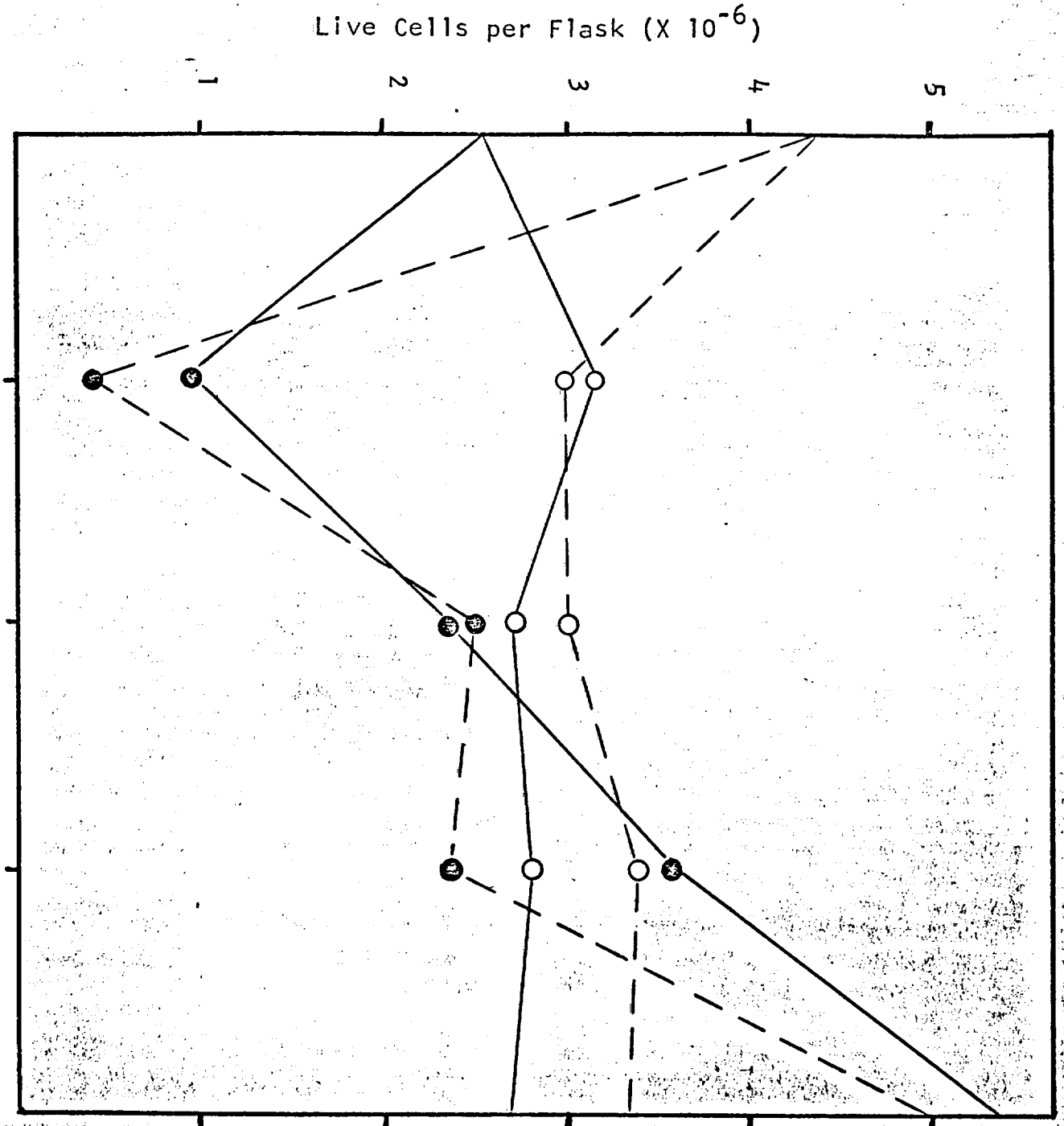
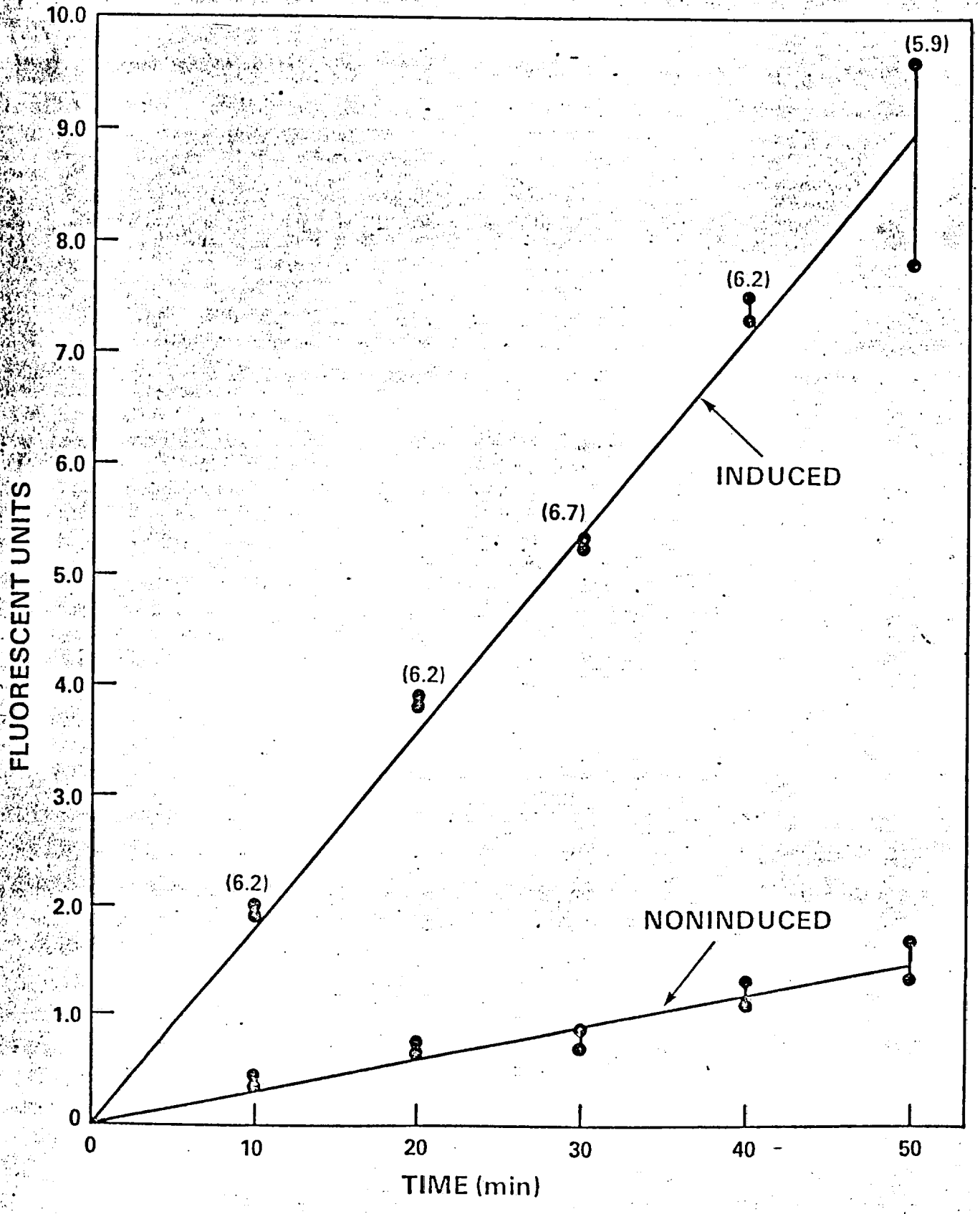


Figure 3

Figure 4



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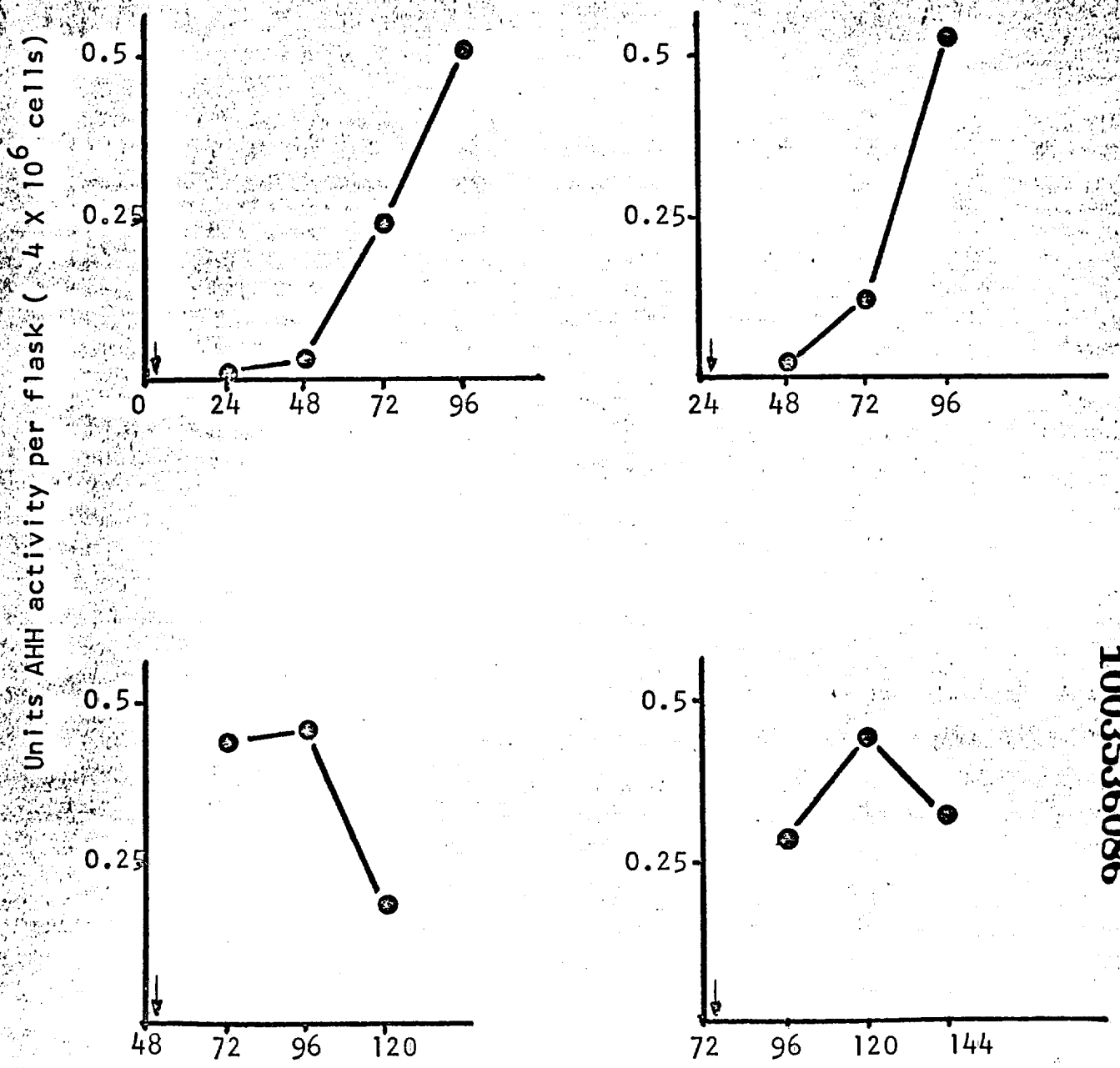
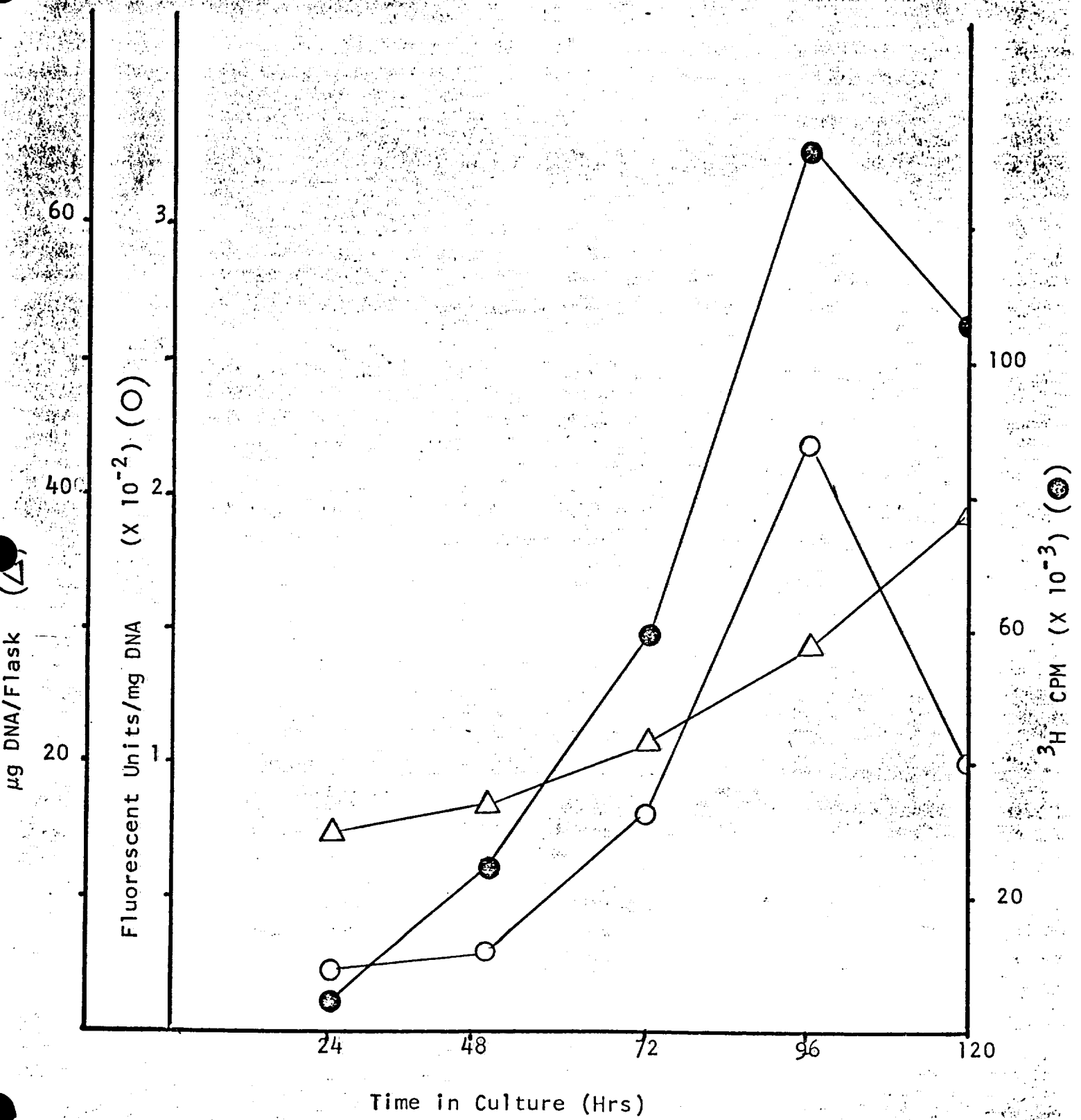


TIME OF INDUCTION WITH MCA

Figure 6



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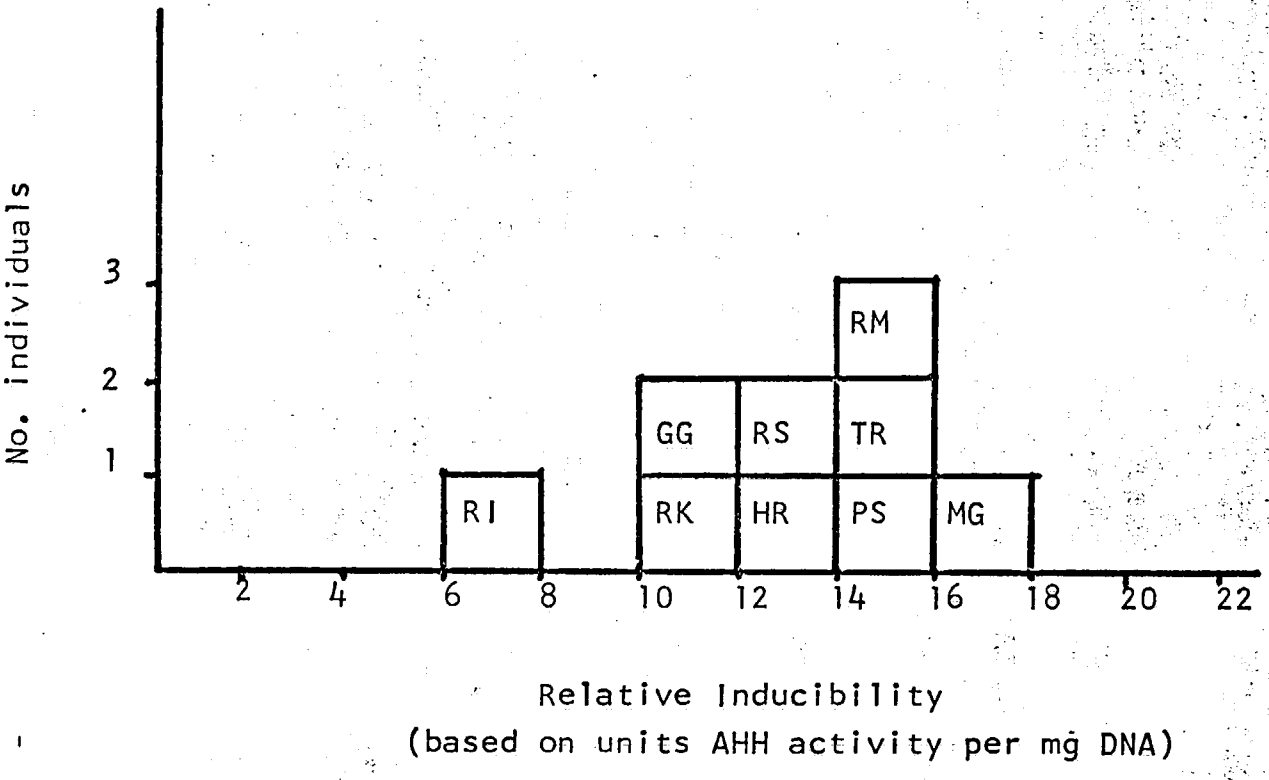

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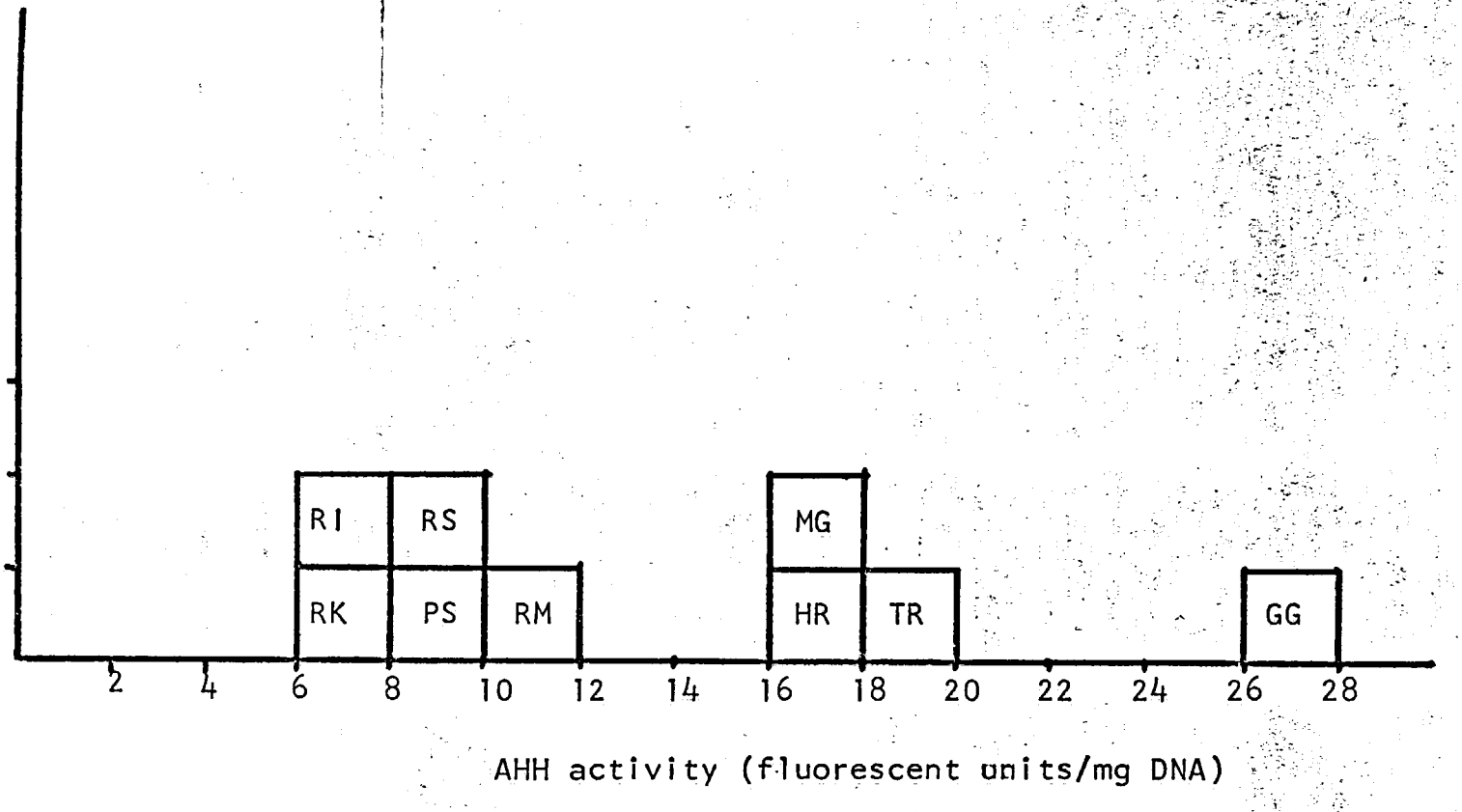

Figure 9.

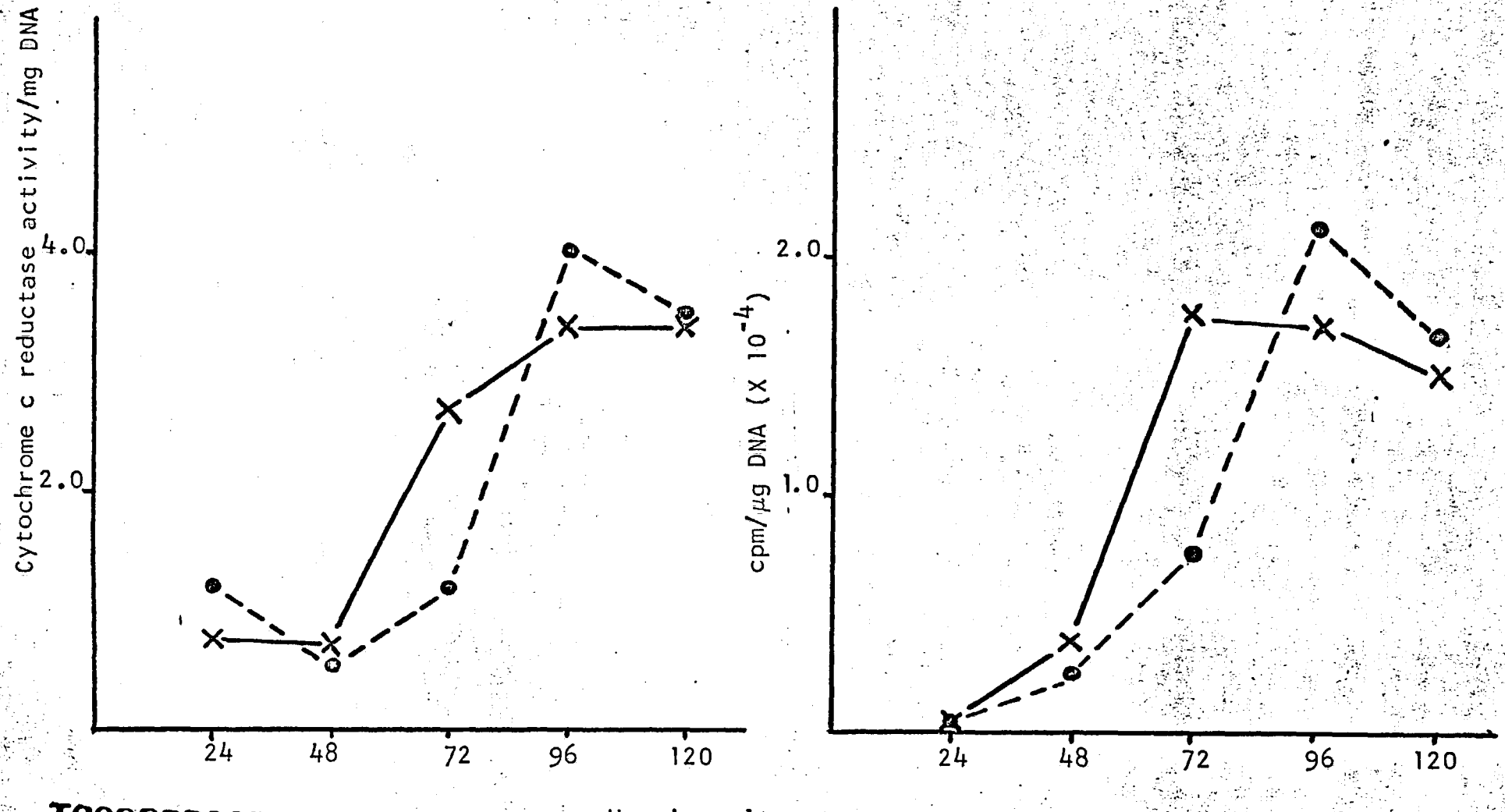

Figure 10